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In vivo evidence that the *nusA* and *infB* genes of *E. coli* are part of the same multi-gene operon which encodes at least four proteins

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Previous work has shown that the *Escherichia coli nusA* gene codes for a protein which regulates transcription termination. The 16.0-kb *Eco*RI DNA fragment that includes the *nusA* gene, codes for at least eight bacterial proteins of mol. wts. 48 000 (*argG*), 21 000 (p21), 64 000 (*nusA*), 120 000 (IF2α)-(*infB*), 91 000 (IF2β)(*infB*), 15 000 (p15), 10 000 (*rpsO*) and 85 000 (*pnp*). We have constructed several deletion and fusion derivatives from this cloned DNA and examined *in vivo* the structure and expression of these genes. First, the promoter functional *in vivo* for the *nusA* gene was mapped at ~800 bp upstream of the *nusA* structural gene. Second, the synthesis of five proteins, p21, NusA, IF2α, IF2β (and p15) proteins, was affected by the deletion of the *nusA* promoter. Third, these same five proteins were hyperproduced after fusion of the DNA fragment to the λ p_L promoter. In addition, subcloning experiments revealed that the p15 gene is expressed by the read-through transcription from the *infB* gene. These results lead us to conclude that the genes coding for the p21, NusA, InfB (IF2α and IF2β), and p15 proteins form a single-transcriptional unit ('*nusA-infB* operon') *in vivo* and that *rpsO* and *pnp* genes do not belong to the same operon. The *in vivo* attenuation site of this operon is described.

Key words: E. coli/hyperproduction/*infB*/*nusA*/operon

Introduction

The *nusA* protein of *Escherichia coli* has been implicated in the regulation of transcription anti-termination in bacteriophage λ (Friedman and Gottesman, 1983) because a mutation in it, *nusA1*, abolishes or reduces anti-termination activity of the λ *N* gene product (Friedman, 1971; Friedman and Baron, 1974). Greenblatt and his co-workers, however, have presented *in vitro* experiments suggesting that the NusA protein, in the absence of *N* protein function, can act as a termination factor at the t_{R2} termination site of λ (Greenblatt *et al.*, 1981). Furthermore, we isolated conditionally lethal amber mutations that map in the *nusA* gene, and showed that these *nusA*-deficient mutants allowed the growth of λN^- phages (Nakamura and Uchida, 1983). These observations suggest that the primary role of the NusA protein is to mediate bacterial transcription termination, and that the function of λ *N* is to overcome this termination. Additional data suggest that the NusA protein participates in the regulation of transcription termination of various *E. coli* genes and several other bacteriophages (Kung *et al.*, 1975; Zarucki-Schulz *et al.*, 1979; Kassavetis and Chamberlin, 1981; Kingston and Chamberlin, 1981; Farnham *et al.*, 1982).

The *nusA* gene is located at 69 min on the *E. coli* chromosome (Bachmann, 1983; Friedman and Baron, 1974). Recent cloning experiments revealed that the 16.0-kb *Eco*RI DNA fragment that includes the *nusA* gene codes for at least eight bacterial proteins of mol. wts. 48 kd, 21 kd, 64 kd, 120 kd, 91 kd, 15 kd, 10 kd and 85 kd. Of these proteins, the 64-kd and 48-kd proteins are the *nusA* and *argG* gene products, respectively (Kurihara and Nakamura, 1983), the 85-kd protein is the *pnp* gene product (Portier *et al.*, 1981) while the 10-kd protein is the ribosomal protein S15 (Portier, 1982). The 120-kd and 91-kd proteins are translation initiation factor IF2α and IF2β, respectively (Plumbridge *et al.*, 1982). Our previous observations and those of Plumbridge *et al.* (1982) indicated that the IF2α and IF2β are coded by the same gene (*infB*) (Kurihara and Nakamura, 1983; Plumbridge and Springer, 1983) and that the gene order is (*argG*, p21)-*nusA*-*infB*-p15-*rpsO*-*pnp*.

The 2430-bp DNA sequence of the *nusA* gene and its flanking region has been determined recently (Ishii *et al.*, 1984a, 1984b). The DNA sequence implied the possibility that the p21, *nusA* and IF2α genes are co-transcribed, in this order, from the promoter located at ~800 bp upstream of the structural gene for *nusA*. However, Plumbridge and Springer (1983) showed that the *infB* gene could be expressed by its own promoter before the *infB* gene. Furthermore, it has not been shown whether genes for p15, *rpsO* and *pnp* are part of the same operon as *nusA* or not. To answer these questions, we have carried out an *in vivo* analysis of the structure and expression of the *nusA* operon of *E. coli*. No *in vivo* results on the structure of this operon have been reported previously.

Results

Gene organization in the vicinity of nusA

Eight proteins are known to be coded by the 16.0-kb *Eco*RI DNA fragment cloned onto plasmid pYN81 (Figure 1) (Portier *et al.*, 1981; Plumbridge *et al.*, 1982; Portier, 1982; Kurihara and Nakamura, 1983; Plumbridge and Springer, 1983). The *nusA* gene is located in the middle of the bacterial fragment, and is transcribed counterclockwise with respect to the *E. coli* genetic map (i.e., rightward in Figure 1) (Kurihara and Nakamura, 1983; Plumbridge and Springer, 1983). The DNA segment upstream of the *nusA* gene encodes for two proteins of mol. wt. 48 kd *argG* and p21 (Kurihara and Nakamura, 1983). Genomic arrangement of these two genes was investigated by studying the DNA structure and gene products of the pYN87 plasmid. First, a restriction enzyme map of the bacterial insert was constructed by partially digesting the end-labeled linear pYN87 DNA. Then, the 1.9-kb *Pst*I fragment encoding the N terminus of NusA was recloned in pBR322 in different orientations (pSM11 and pSM12, see Figure 2). The gene products of pSM11 and pSM12 were analyzed by labeling proteins synthesized in minicells carrying these plasmids. As shown in Figure 3 lanes 2 and 3, pSM11 produced p21 and a 55-kd protein, whereas pSM12 produced

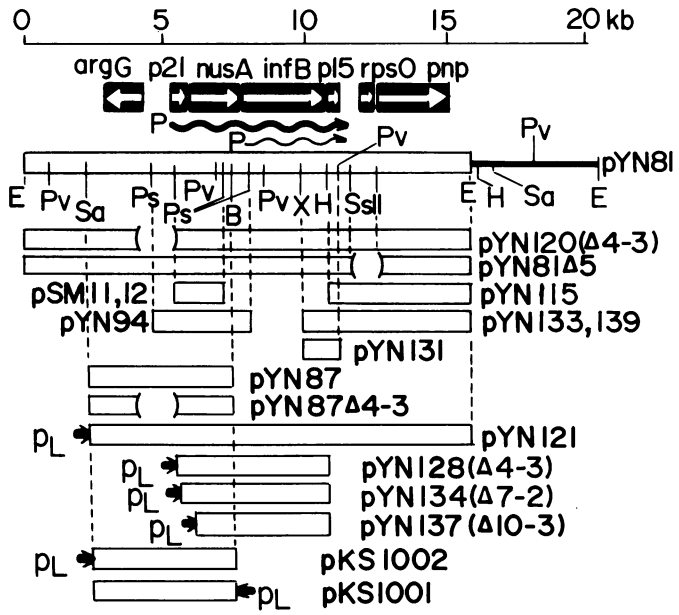

Fig. 1. Organization of bacterial genes carried on the pYN81 plasmid and its subclones. Construction of these plasmid DNAs is described in Materials and methods. The structures of some of these plasmid DNAs are shown in Figure 2. Closed boxes indicate the locations of genes encoding proteins and arrows indicate direction of transcription. Open boxes indicate bacterial DNA fragments and bold bars indicate pBR322 DNA. Deleted DNA segments are enclosed by brackets. The last six plasmids (pYN121 through pKS1001) are hyperproducing plasmids containing the p_L promoter segment of λ (450-bp *Bgl*II-*Hpa*I λ DNA; as marked by bold arrows). The pBR322 portion of DNA for plasmids other than pYN81 is not indicated because the insertion site in these plasmids differs from pYN81. 'P' represents a promoter and wavy arrows indicate a transcriptional unit and its direction as deduced in the text. The scale is in kb as measured from the *Eco*RI site of the DNA insert. E = *Eco*RI, Sa = *Sal*I, B = *Bgl*II, X = *Xho*I, H = *Hind*III, SsII = *Sst*II, Ps = *Pst*I, Pv = *Pvu*II. (All *Pst*I sites are not shown here but only those located on the 6.3-kb *Sal*I-*Pvu*II DNA segment are indicated.)

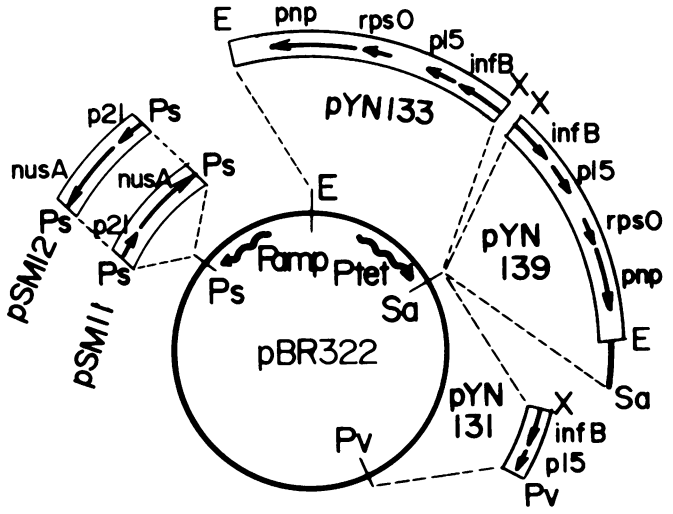

Fig. 2. Structure of chimeric plasmids. Insertion sites and orientations of bacterial DNA fragments of plasmids are indicated. Arrows represent genes and their orientations carried on the bacterial DNA segments. Wavy arrows marked by 'Ptet' and 'Pamp' represent transcriptions initiated from the tetracycline and ampicillin promoters, respectively. Other symbols are those described in Figure 1.

p21 and a 45-kd protein. These results can be explained by assuming that the truncated *nusA* gene is fused in-phase at its *Pst*I site with pBR322 DNA to give rise to a polypeptide containing 60 (pSM11) or 9 (pSM12) extra amino acids (Sutcliffe, 1978). Bacteria carrying the pSM11 and pSM12 plasmids produced p21 but neither produced the 48-kd protein nor complemented the *argG* mutation, consistent with the assignment of the 48-kd protein as the *argG* gene product (Table I). Therefore, the gene order on the *Sal*I-*Bgl*II fragment appears to be *argG*(48-kd)-p21-*nusA*. The p21 protein may correspond to the open reading frame encoding a protein of 15 kd deduced by DNA sequence (Ishii *et al.*, 1984b).

Complementation studies of various *nusA* mutations by truncated-*nusA* plasmids are shown in Table I. pSM11 and pSM12 were able to complement the *nusA1* mutation for λ phage growth although they encoded only the N-terminal two-thirds of the NusA protein. However, they failed to complement the amber mutations *nusA3* and *nusA4* and restore viability at high temperature. The difference of complementation activity of the truncated NusA for the *nusA1* and *nusA* amber mutations is discussed below.

Hyperproduction of NusA and the neighboring gene products

To investigate the direction of transcription and the transcriptional unit of the genes located in the vicinity of the *nusA* locus, the bacterial DNA fragment was fused to the p_L promoter of λ, and the hyperproduction of gene products was analyzed with plasmid pKS1001 (carrying the *nusA* gene in opposite transcriptional orientation relative to the p_L promoter) and pKS1002 (carrying the *nusA* gene in the same transcriptional orientation relative to the p_L promoter). These plasmids were transformed into HB101 cells carrying plasmid pNT203 encoding for the *cI857* repressor. Upon heat induction, ArgG protein (48 kd) accumulated in pKS1001-bearing cells to ~15% of total protein, while the synthesis of the p21 and 61-kd proteins was not affected (Figure 4a). [The 61-kd protein is the truncated NusA protein produced by the *Sal*I-*Bgl*II DNA segment which lacked the C terminus 100-bp sequence of the *nusA* gene (Kurihara and Nakamura, 1983).] On the other hand, the 61-kd and p21 proteins were hyperproduced in pKS1002-bearing cells while the 48-kd protein was not (Figure 4b). Therefore, we concluded that the gene coding for the p21 protein is transcribed in the same direction as the *nusA* gene, and that the *argG* gene is transcribed in the opposite direction (see Figure 1).

We then constructed plasmid pYN121 whose bacterial insert encoding the downstream genes of *nusA* (13.7-kb *Sal*I-*Eco*RI fragment) is joined to p_L. Heat induction of HB101[pNT203] carrying pYN121 triggered hyperproduction of four proteins, 120 kd (IF2α), 91 kd (IF2β), 64 kd (NusA) and p15, although the induction of p21 synthesis was not obvious in this experiment (Figure 4c). Two hours after induction, the intracellular contents of the IF2α, IF2β, NusA and p15 proteins increased to 4%, 2%, 5% and 1% of total intracellular protein, respectively. No appreciable overproduction of the S15 (10-kd) and Pnp protein (85-kd) was observed. These results indicate that the genes coding for the p21, NusA, IF2α, IF2β and p15 proteins are transcribed in the same direction.

Deletion mutants lacking the nusA promoter

To isolate deletion mutants lacking the *nusA* promoter, nine deletion plasmids were constructed from pYN87 DNA after *Bal*31 digestion. (The structures of these plasmids were determined by electrophoresis of restriction digests on agarose and

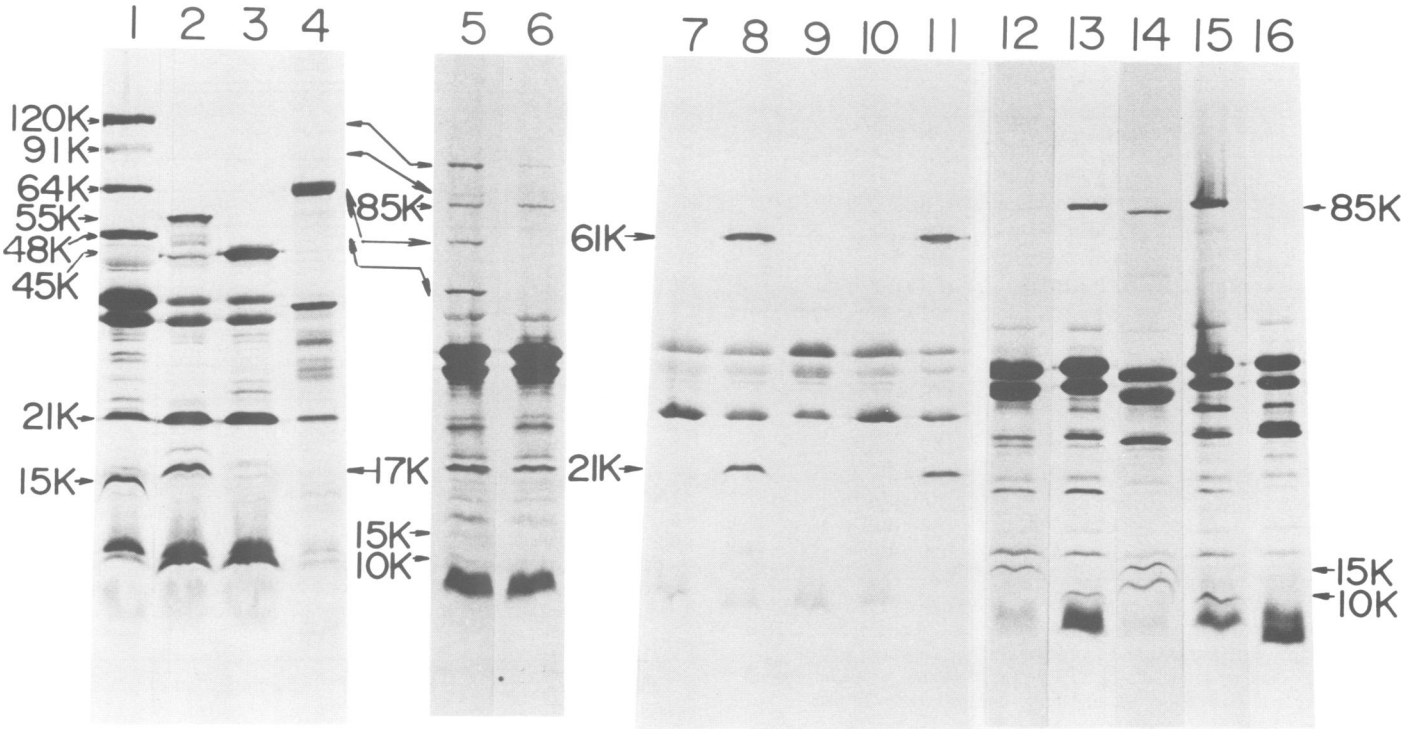

Fig. 3. Autoradiograph of SDS-polyacrylamide gel electrophoresis of proteins synthesized by minicells carrying a chimeric plasmid. Experimental procedures and conditions are described in Materials and methods. **Lane 1**, pYN81Δ5; **lane 2**, pSM11; **lane 3**, pSM12; **lane 4**, pYN94; **lane 5**, pYN81; **lane 6**, pYN120; **lane 7**, pYN87Δ3-1 (see Figure 5); **lane 8**, pYN87Δ3-2 (see Figure 5); **lane 9**, pYN87Δ4-3 (see Figure 5); **lane 10**, pYN87Δ5-1 (see Figure 5); **lane 11**, pYN87Δ5-5 (see Figure 5); **lane 12**, pYN131; **lane 13**, pYN115; **lane 14**, pYN139; **lane 15**, pYN133; **lane 16**, pBR322. Labeled protein bands indicated by arrows are bacterial proteins encoded by the cloned DNA. 61 K, 55 K and 45 K are truncated *nusA* gene products. (The 17 K band seen in lane 2 may be a truncated β-lactamase as judged by the size.) 120 K = IF2α, 91 K = IF2β, 64 K = NusA, 48 K = ArgG, 10 K = S15. Electrophoresis was carried out on slab gels containing 5% stacking gels and separation gels with 10−20% linear gradient. The following proteins were used as mol. wt. standards: bovine serum albumin (68 kd); bovine pancreatic trypsin (24 kd); egg white lysozyme (14.4 kd); *E. coli* RNA polymerase (subunit β′, 160 kd; β, 150 kd; σ, apparent mol. wt. 86 kd; α, 40 kd).

polyacrylamide gels and are presented in Figure 5.) Table I shows results of the complementation analysis carried out with these deletion plasmids. Two plasmids containing deletions smaller than Δ4-3 complemented *nusA1*, *nusA3*(am) and *nusA4*(am) mutations, whereas the other seven plasmids containing deletions larger than Δ5-5 did not. The end points of deletions Δ5-5 and Δ4-3 are in positions about +30 and +100 bp starting from the *Pst*I site, respectively (see Figure 5). Proteins synthesized by these deletion plasmids were examined in the minicell system. As shown in Figure 3 lanes 7−11, synthesis of p21 and the 61-kd protein (truncated-NusA) was not affected by Δ3-2 or Δ5-5; however, the plasmid carrying Δ4-3, Δ5-1 or Δ3-1 was unable to produce either the p21 or the 61-kd protein. Therefore, we conclude that the gene encoding the p21 protein forms an operon with *nusA*, and that the promoter for this operon is located between +30 and +100 bp positions from the *Pst*I site defined by Δ5-5 and Δ4-3.

Structure of the nusA operon

Since pYN87 DNA lacked DNA sequences downstream of the *Bgl*II site mapping in the C terminus of the *nusA* gene, the deletion Δ4-3 was transferred onto the pYN81 DNA to examine whether expression of the *infB* and its downstream genes is affected by the promoter deletion. The pYN120 DNA is structurally equivalent to the pYN81 DNA except that the former contains the Δ4-3 deletion. As shown in Figure 3 lanes 5 and 6, the Δ4-3 deletion affected synthesis of five proteins: synthesis of the NusA (64 kd) and the ArgG (48 kd) proteins was abolished and that of the IF2α, IF2β and presumably p15 proteins was reduced to 5% or less of the pYN81 level. Synthesis of the *pnp* product (85 kd) and the S15 protein was not affected. (The p21 protein band overlapped with the background proteins of minicells in Figure 3 lanes 5 and 6.) Disappearance of 48-kd protein synthesis was due to the cleavage of *argG* at *Kpn*I site(s) but not due to the deletion of the *nusA* promoter. These results indicate that the promoter specified by Δ4-3 controls not only *nusA* but the *infB* and probably p15 protein genes. However, the residual 5% synthesis of IF2α protein suggested that an internal weak promoter is located before the *infB* gene (see Figure 1). Minicells carrying the pYN115 DNA which contains *rpsO* and *pnp* genes synthesized the S15 and Pnp proteins but did not synthesize the p15 protein (Figure 3 lane 13). However, those carrying the pYN131 DNA, which contains the 1.17-kb *Xho*I-*Pvu*II DNA fragment, synthesized the p15 protein (Figure 3 lane 12), indicating that the p15 gene is located close to the *infB* gene. Consistent with this, minicells carrying the pYN139 DNA, which contains the 6.0-kb *Xho*I-*Eco*RI DNA segment, synthesized the p15 protein in addition to the S15 and Pnp proteins (Figure 3 lane 14). However, minicells carrying the pYN133 DNA, which contains the identical *Xho*I-*Eco*RI DNA segment in opposite orientation, synthesized the S15 and Pnp proteins but did not synthesize the p15 protein (Figure 3 lane 15). Therefore, it was suggested that the synthesis of the p15 protein observed with plasmids pYN131 and pYN139 was mainly due to the read-through transcription from the *tet*^{r}

Table I. Complementation tests of several *nusA* mutations with chimeric plasmids

Plasmid	Mutation			
	nusA1[a]	*nusA3*(am)[b]	*nusA4*(am)[b]	*argG*[c]
pBR322	−(0.006)	$-(3.2 \times 10^{-7})$	$-(1.8 \times 10^{-8})$	−
pYN81	+	+(1.0)	+(1.0)	+
pYN87	+(14.2)	+(1.0)	+(1.0)	+
pYN94	+(11.2)	+(1.0)	+(1.0)	−
pSM11	+(18.8)	$-(2.0 \times 10^{-3})$	$-(1.3 \times 10^{-3})$	−
pSM12	+(21.4)	$-(1.1 \times 10^{-2})$	$-(8.2 \times 10^{-3})$	−
Δ3-2	+	+(1.0)	+(1.0)	−
Δ5-5	+	+(1.0)	+(1.0)	−
Δ4-3	−	$-(7.2 \times 10^{-4})$	$-(3.3 \times 10^{-4})$	−
Δ5-1	−	$-(6.8 \times 10^{-4})$	$-(1.3 \times 10^{-3})$	−
Δ7-2	−	$-(4.8 \times 10^{-4})$	$-(2.5 \times 10^{-4})$	−
Δ10-3	−	$-(2.4 \times 10^{-4})$	$-(2.4 \times 10^{-4})$	−
Δ3-1	−	$-(6.0 \times 10^{-4})$	$-(1.8 \times 10^{-4})$	−
Δ9-1	−	$-(4.2 \times 10^{-4})$	$-(1.8 \times 10^{-4})$	−
Δ8-1	−	$-(3.5 \times 10^{-8})$	$-(2.0 \times 10^{-8})$	−

Cells were transformed with plasmid DNA, selecting for Tet[r] (pSM11, pSM12 and pYN94) or Amp[r] (the other plasmids) colonies at 30°C. These transformants were purified and scored on the same selective plates containing 50 μg/ml ampicillin or 20 μg/ml tetracycline. + means growth of phage or cell (complementation), and − means no growth (no complementation).

[a]Growth of λ*cI71* phage on cells of YN3006 at 42°C. λ phage growth was examined by cross-streaking on an EMB plate or by assaying the phage yield. Numbers enclosed by parentheses are the number of progeny phage released per infected cell.

[b]Growth of cells of YN2359 or YN2360 at 42°C. Numbers enclosed in parentheses are the frequency of apparent temperature-resistant 'reversion' at 42°C.

[c]Growth of cells of YN3006 on arginine-free minimal plates.

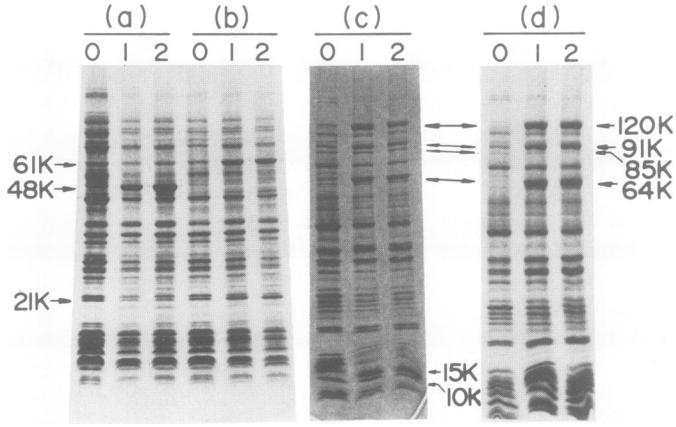

Fig. 4. Hyperproduction of proteins encoded by the *nusA-infB* operon. Cultures (10 ml) of HB101[pNT203] carrying pKS1001 **(a)**, pKS1002 **(b)**, pYN121 **(c)** or pYN128 **(d)** were grown at 30°C and transferred to 42°C (0 h). Samples (4 x 10^8 cells) were taken at the time indicated (h). Whole cell proteins were precipitated with TCA, rinsed with acetone, and subjected to SDS-gel electrophoresis. Gel patterns stained with Coomassie brilliant blue are presented. Experimental procedures and conditions are described in Materials and methods.

promoter of pBR322 beyond the C-terminal *infB* segment.

To confirm that the structural genes themselves for p21, NusA, IF2α and IF2β carried on pYN120 DNA were not damaged by the Δ4-3 deletion, we isolated the *Hind*III fragment coding these sequences from pYN120, and fused it to the p_L promoter (pYN128, see Figure 1). As shown in Figure 4d, HB101[pNT203] cells carrying pYN128 hyperproduced

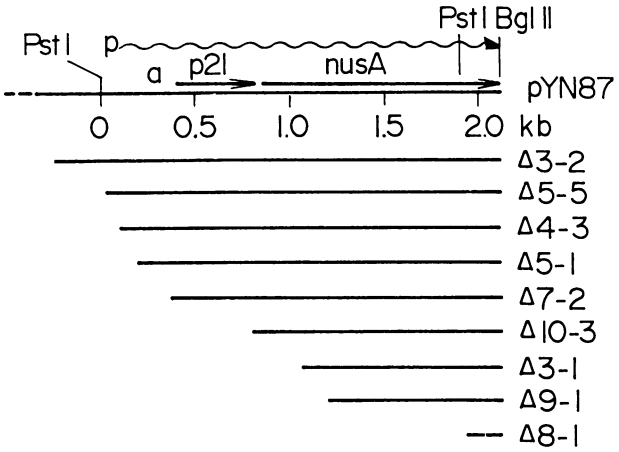

Fig. 5. Structure of deletions on the bacterial DNA segment carried on pYN87 DNA. These deletions were constructed by the *Bal*31 exonuclease digestion. Details are described in Materials and methods. Bold bars indicate bacterial DNA, and arrows indicate location and orientation of genes. 'P' and wavy line indicate a promoter and transcriptional unit as deduced in the text, respectively. 'a' indicates an attenuator.

Table II. Effect of λ *N* protein on the hyperproduction of p21 and NusA proteins

Plasmid		Hyperproduction	
		p21	NusA
pKS1002	N^+	+	+
	N^-	−	−
pYN128 (Δ4-3)	N^+	+	+
	N^-	−	−
pYN134 (Δ7-2)	N^+	+	+
	N^-	+	+
pYN137 (Δ10-3)	N^+	−	+
	N^-	−	+

Plasmids were transformed into HB101 cells containing pNT203(N^+) or pNT204(N^-). Hyperproduction of the p21 and NusA proteins was examined as described in Materials and methods and Figure 4, or by pulse-labeling the proteins with [^{14}C]amino acid mixture upon heat induction and subsequent SDS-gel analysis. + means hyperproduction and − means no hyperproduction.

the 120-kd (IF2α), 91-kd (IF2β) and 64-kd (NusA) proteins upon heat induction. Though the enrichment of p21 protein was not observed in Figure 4d, the pulse-labeling experiments indicated that the rate of synthesis of p21 protein was increased by heat induction, suggesting rapid degradation of the p21 protein under these experimental conditions (data not shown). The contents of IF2α, IF2β and NusA reached 5.7%, 4% and 6.5% of total intracellular protein, respectively, 1 h after temperature shift-up.

In vivo evidence for the attenuator in the nusA leader sequence

Hyperproduction of the NusA and other proteins with plasmids pKS1002, pYN121 and pYN128 was successful in the presence of the *N* protein of phage λ (pNT203; *cI857-N*$^+$). No proteins were hyperproduced when the pNT203 was replaced by the N^- derivative of pNT203 (pNT204; *cI857-N*$^-$) (Table II). The pYN128 DNA lacked the *nusA* promoter removed by the Δ4-3 deletion and the 450-bp p_L segment was directly joined to the *nusA-infB* sequence. Two DNA fragments containing larger deletions (Δ7-2 and Δ10-3) than

Δ4-3 were joined to the p_L segment (pYN134 and pYN137) and synthesis of the p21 and NusA proteins was examined upon heat induction. As shown in Table II, the NusA protein was hyperproduced with both plasmids pYN134 and pYN137 even in the absence of λ *N* gene. These observations indicate that a termination signal which is sensitive to λ *N* protein is located at the 100(Δ4-3)-350(Δ7-2) bp position from the *Pst*I site. Observations that the deletion Δ10-3 eliminated hyperproduction of the p21 protein but the Δ7-2 did not (Table II) suggested that the p21 gene is located at the 350(Δ7-2)-800(Δ10-3) bp position from the *Pst*I site.

Discussion

The experiments presented here demonstrated *in vivo* that the *nusA* gene forms an operon with the *infB* gene and the two genes coding for the p21 and p15 proteins. The gene order is p21-*nusA-infB*-p15, and the direction of transcription is counterclockwise with respect to the *E. coli* genetic map. The promoter was identified *in vivo* at a position ~800 bp upstream from the coding sequence of *nusA* (30−100 bp position from the *Pst*I site). Evidence for the *nusA-infB* operon was based on the following observations. (i) The plasmid deleting the *nusA* promoter failed to produce the p21 and NusA proteins, and produced the IF2 and p15 proteins at 5% of the level of the parental plasmid in minicells. (ii) The expression of the p15 gene was dependent on the read-through transcription from the *infB* gene. (iii) The plasmid carrying the p21, *nusA*, *infB* and p15 genes under control of the p_L promoter of λ hyperproduced the p21, NusA, IF2α, IF2β and p15 proteins upon heat induction. The 5% residual synthesis of the IF2 and p15 proteins by the promoter-deletion plasmid suggested the existence of an internal weak promoter before the *infB* gene. This may correspond to the promoter reported by Plumbridge and Springer (1983).

Ishii *et al.* (1984b) found an open reading frame encoding a protein of 15 kd just upstream of *nusA*. It corresponds to the p21 protein we identified in this communication. In addition, they found a gene coding for a minor form of initiator fMet-tRNA (*metY*) between the promoter and the p21 gene, suggesting that the *metY* gene is part of the *nusA-infB* operon (Ishii *et al.*, 1984b). The function of the p21 and p15 proteins is not known. The genes *rpsO* and *pnp* are located downstream from the p15 gene and are transcribed in the same direction as the *nusA-infB* operon. However, these two genes do not belong to the same operon because the *nusA* promoter deletion (Δ4-3) or the p_L promoter fusion did not affect the synthesis of the S15 or Pnp proteins. Furthermore, synthesis of the p15 protein was abolished in the pYN133 plasmid whereas those of S15 and Pnp were not affected.

Hyperproducer plasmids were constructed by fusing the *nusA-infB* segment to the λ p_L promoter (pYN121 and pYN128, see Figure 1). The presence of λ *N* protein is essential to induce hyperproduction of gene products from pYN121 or pYN128; no proteins were hyperproduced when the pNT203(*cI857-N*$^+$) was replaced by the pNT204(*cI857-N*$^-$) plasmid. Deletion and fusion experiments demonstrated that a termination signal that can be overcome by the function of *N* protein is located at the leader sequence of the *nusA* operon (see Table II). This may correspond to the ρ-independent termination signals deduced by the DNA sequence (Ishii *et al.*, 1984b). Therefore, it can be suggested that the *nusA-infB* operon is regulated by an attenuator *in vivo*. However, the S15 and Pnp proteins were not hyperproduced by plasmid pYN121 even in the presence of the λ *N* protein (see Figure 4c). This observation implies that the *N*-resistant termination signal is located between the p15 gene and the *rpsO* gene.

Previously we have isolated amber mutants which affected the synthesis of NusA protein (Nakamura and Uchida, 1983). Here we present direct evidence that these mutations occur within the structural gene of *nusA*. The amber mutants (YN2359 and YN2360) transformed with six deletion plasmids Δ4-3 to Δ9-1 exhibited an apparent temperature-resistant 'reversion' at a frequency of $10^{-4}-10^{-5}$ as compared with $10^{-7}-10^{-8}$ for the parental mutant strains (Table I). In contrast, the Δ8-1 deletion plasmid did not bring about such enhancement. The fact that Δ9-1 but not Δ8-1 showed this effect suggested that the wild-type alleles of these amber mutations (formerly called *am3* and *am4*) are located in the structural gene of *nusA* (in the 800-bp segment specified by Δ9-1 and Δ8-1), and that the increased 'reversion' was due to recombination between the host and plasmid DNA sequences. Thus, we propose to rename the *am3* amd *am4* mutations *nusA3*(am) and *nusA4*(am), respectively. These *nusA* amber mutations cannot be complemented by the 1.9-kb *Pst*I fragment encoding the N-terminal two third polypeptide of NusA, in contrast to the results that the same *Pst*I fragment is able to complement the *nusA1* mutation (pSM11 and pSM12, see Table I). These contradictory observations can be explained if one assumes that the 2/3-NusA protein can no longer function as a transcriptional termination protein, and thus is unable to complement the *nusA* amber mutations, but still harbors the capacity to antiterminate the transcription of λ at the termination signal, thus complementing the *nusA1* mutation. This capacity may be due to the ability of the 2/3-NusA protein either to compete with the *nusA1* protein for binding to the RNA polymerase (thus preventing transcription termination) or with its ability to interact functionally with the *N* protein of phage λ.

Materials and methods

Bacterial and phage strains

Bacterial strains (*E. coli* K12) used are as follows: YN3006[F$^-$ *gal-1 gal-2 lac rpsL argG nusA1* Su$^-$], YN2359[F$^-$ *sup-126 nusA3*(am) *metB trpE9829*(am) *tyr*(am)] (Nakamura and Uchida, 1983), YN2360[F$^-$ *sup-126 nusA4*(am) *metB trpE9829*(am) *tyr*(am)] (Nakamura and Uchida, 1983), P678-54[F$^-$ *thr leu lacY minA T6^s gal minB rpsL thi* Su$^+$] (Adler *et al.*, 1967), and HB101[F$^-$ *hsdS20*(r_B^-, m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*] (Maniatis *et al.*, 1982). YN2359 and YN2360 strains carry amber mutations in the *nusA* gene (Nakamura and Uchida, 1983). Since these strains carry a temperature-sensitive amber suppressor (*sup-126*; Nagata and Horiuchi, 1973), both amber mutants grow at 30°C but not at 42°C. The phage used was λ*cI71*.

Media, buffers and chemicals

The broth and agar media used were described previously (Saito and Uchida, 1977). Minimal medium was medium E (Vogel and Bonner, 1956) or ML (Curtiss, 1965) with appropriate supplements. Buffers and chemicals used were generally those described previously (Nakamura, 1980).

Restriction enzyme analysis of DNA

Restriction enzyme digestions were performed as described previously (Maniatis *et al.*, 1982). DNA fragments were analyzed by electrophoresis on 0.7% agarose or 3.5% polyacrylamide gels in the Tris/borate buffer described previously (Nakamura, 1980). DNA size markers used were *Eco*RI/*Hind*III single or double digests of λ DNA, or *Alu*I or *Eco*RI/*Hin*fI double digests of pBR322 DNA.

Plasmids

The structures of the chimeric plasmids used are shown in Figures 1 and 2. Plasmid pYN81 (pBR322 carrying the 16.0-kb *Eco*RI fragment) and its subclone derivative (pYN87) have been described previously (Kurihara and Nakamura, 1983). Plasmids pSM11 and pSM12 have a 1.9-kb *Pst*I insert, which includes the N terminus of the *nusA* gene, inserted at the *Pst*I site of pBR322 in

opposite orientations. Plasmid pYN94 carries the same fragment and the adjacent *Pst*I fragments of 0.74 and 0.95 kb. These three plasmids were constructed by subcloning the complete (pSM11 and pSM12) or partial (pYN94) *Pst*I digests of pYN81 onto *Pst*I-cut pBR322.

To isolate deletion plasmids lacking the *nusA* promoter, pYN87 DNA was cleaved at its *Kpn*I sites, and digested with exonuclease *Bal*31 for various times. T4 DNA polymerase was used to fill in the ends, and *Hind*III linkers were ligated as described by Maniatis *et al.* (1982). Nine deletion plasmids were isolated. One of the deletions, Δ4-3, was transferred onto pYN81 plasmid DNA by replacing the wild-type *Sal*I-*Pvu*II fragment with the mutant fragment *in vitro*, giving rise to plasmid pYN120.

The 14.4-kb *Sal*I fragment of pYN81 was inserted at the *Sal*I site of pTK32 to give rise to plasmid pYN121 and the 5.45-kb *Hind*III fragment of pYN120 was inserted into *Hind*III site of pTK32 to give rise to plasmid pYN128. [The pTK32 DNA contains the 450-bp *Bgl*II-*Hpa*I segment carrying the sequence for λ p_L promoter and the 5′ half of the *N* gene inserted at the *Hind*III site of pBR322 in the same transcriptional direction as *tet*^{r} (T.Kurihara and Y.Nakamura, unpublished).] Plasmids pYN134 and pYN137 carry the p_L promoter segment upstream of the *nusA* segment containing deletions Δ7-2 and Δ10-3, respectively. Plasmids pKS1001 and pKS1002 are another set of hyperproducer plasmids: they contain the *Sal*I-*Bgl*II bacterial DNA segment derived from pYN87 in opposite orientation downstream from the p_L promoter carried on pNT38. [pNT38 is a pBR322 derivative carrying the 450-bp p_L DNA segment flanked by the *Bgl*II and *Hpa*I sites inserted at the *Bam*HI site (N. Tsurushita *et al.*, unpublished).] The *Sal*I-*Bgl*II fragment was inserted at the *Sal*I or *Sal*I and *Pvu*II sites of pNT38 with linkers. Expression of p_L from these hyperproducer plasmids is regulated by the *cI857* repressor carried on pNT203. The pNT203 plasmid is a derivative of pSC101 and carries a *cI857-N*$^+$ gene segment of λ (N.Tsurushita *et al.*, unpublished). The pNT204 plasmid is a *cI857-N*$^-$ derivative of pNT203 (N.Tsurushita *et al.*, unpublished). pYN115 DNA was constructed by subcloning the 5.1-kb *Eco*RI-*Hind*III DNA fragment encoding the S15 and Pnp proteins from pYN81 onto pBR322 DNA. The 6.0-kb *Xho*I-*Eco*RI DNA fragment encoding the p15, S15 and Pnp proteins was inserted at the *Eco*RI/*Sal*I or *Sal*I site of pBR322 in opposite orientation, giving rise to plasmids pYN133 and pYN139, respectively. The 1.7-kb *Xho*I-*Pvu*II DNA fragment encoding p15 was subcloned onto pBR322, giving rise to plasmid pYN131.

Hyperproduction

Hyperproducer plasmids were transformed into HB101 cells containing pNT203. Plasmid pNT203 is compatible with pBR322 derivatives, and they can be maintained in the same host by selecting for Amp^r and Tet^r. Cells bearing these plasmids were grown overnight at 30°C in L broth supplemented with 25 μg/ml ampicillin and 10 μg/ml tetracycline. A portion was diluted 20-fold into L broth without antibiotics and grown at 30°C to a cell density of 4 x 10^8/ml. The culture was then shifted to 42°C, and samples were removed at 1 h intervals and tested for protein overproduction.

Analysis of gene products in minicells

A minicell-producing strain (P678-54) carrying a given plasmid was grown in medium ML with appropriate supplements. Minicells were purified according to the procedure previously described (Nakamura *et al.*, 1983). Proteins synthesized in minicells were labeled with 1 μCi/ml [^{14}C]amino acid mixture (NEC-445, New England Nuclear, USA), precipitated washed with 10% TCA, and rinsed with acetone. These labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis as described previously (Nakamura *et al.*, 1983). Gels were stained with Coomassie brilliant blue, dried and exposed to Kodak X-ray film for autoradiography.

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